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Short running head: Serum MicroRNAs in Small Bowel NET

ABSTRACT

Objective: To discover serum-based microRNA (miRNA) biomarkers for small bowel neuroendocrine tumors (SBNET) to help guide clinical decisions.

Background: MiRNAs are small non-coding RNA molecules implicated in the initiation and progression of many cancers. MiRNAs are remarkably stable in bodily fluids, and can potentially be translated into clinically useful biomarkers. Novel biomarkers are needed in SBNET to determine disease aggressiveness, select patients for treatment, detect early recurrence and monitor response.

Methods: This study was performed in 3 stages (discovery, validation and a prospective, longitudinal assessment). Discovery comprised of global profiling of 376 miRNA in sera from SBNET patients (n=11) vs. healthy controls (HC; n=3). Up-regulated miRNAs were subsequently validated in additional SBNET (n=33) and HC sera (n=14); and then longitudinally after SBNET resection (n=12), with serial serum sampling (pre-operatively day 0; post-operatively at 1 week, 1 month and 12 months).

Results: Four serum miRNAs (miR-125b-5p, -362-5p, -425-5p and -500a-5p) were significantly up-regulated in SBNET (P<0.05; fold-change >2) based on multiple normalization strategies, and were validated by RT-qPCR. This combination was able to differentiate SBNET from HC with an AUC of 0.951. Longitudinal assessment revealed that miR-125b-5p returned towards HC levels at 1 month post-operatively in patients without disease, whilst remaining up-regulated in those with residual disease (RSD). This was also true at 12 months post-operatively. In addition, miR-362-5p appeared up-regulated at 12 months in RSD and recurrent disease (RCD).

Conclusions: Our study represents the largest global profiling of serum miRNAs in SBNET patients, and the first to evaluate ongoing serum miRNA expression changes after surgical resection. Serum miR-125b-5p and miR-362-5p have potential to be used to detect RSD/RCD.

268 words

Keywords: microRNAs; small bowel; neuroendocrine tumor; biomarkers; recurrent disease; serum.

INTRODUCTION

The incidence of neuroendocrine tumors (NET), more recently renamed as neuroendocrine neoplasia, has risen 5-7 fold over the past three decades, with the majority of them localized within the gastrointestinal tract.[1] Neuroendocrine tumors are now the most common neoplasm of the small intestine, and are the most common type of NET to develop distant metastases.[2, 3] Indeed, whilst the vast majority of small bowel NET (SBNET) are well-differentiated (G1 or G2) lesions, about 90% still have lymph-node metastases,[4] and 45-70% have liver metastases at the time of diagnosis.[5, 6] Radical resection of locoregional disease, using the principles of small bowel-sparing surgery, and resection of associated liver metastases has the potential for favourable long term survival in well-selected patients with SBNET. However, despite complete elimination of macroscopic disease, a substantial number develop recurrent disease. Several risk factors for recurrence have been identified, such as distant abdominal and mesenteric LN metastases, primary tumor multicentricity (present in about 30% of cases), mesenteric tumor deposits and hepatic micrometastases.[7, 8]

Morphologic and functional imaging both have limitations in detecting residual small volume tumor deposits or early stage disease recurrence.[6] Chromogranin A (CgA) is a widely used circulating biomarker for NET, however its clinical value is burdened by impaired sensitivity and specificity, and lack of standardisation of assays used. These shortcomings in the clinical management of SBNET call for novel biomarkers to be developed that have the potential to identify disease, assess effectiveness of treatment, identify those patients who would benefit from adjuvant treatment, and detect early recurrence. Various strategies have been tested, including whole-blood PCR-based multianalyte liquid biopsy or NETest,[9-12] urinary metabolomic phenotyping,[13] serum protein profiling,[14] and circulating microRNAs (miRNAs).[15]

MiRNAs are short (~17-25 nucleotides), non-coding RNA molecules that regulate gene expression at the post-transcriptional level by translational repression or exonucleolytic degradation of target messenger RNAs (mRNAs).[16] MiRNAs control >60% of human protein-coding genes, and each miRNA is able to regulate hundreds of mRNAs. MiRNA expression is altered in various

cancers, both in tissues and the systemic circulation.[17, 18] Dysregulated miRNAs can act either as oncomiRs or tumor suppressors depending on their target genes. Accordingly, altered miRNA expression has been shown to contribute to many different aspects of tumorigenesis and metastasis. Furthermore, miRNAs in the blood of cancer patients have been found to be novel non-invasive biomarkers for diagnosis, prognosis and response to treatment. Importantly, blood miRNAs are remarkably stable after prolonged incubation at room temperature and/or multiple freezing–thawing processes. Consequently, the concept of using circulating miRNAs as a "liquid biopsy" has evolved. Previous studies have identified circulating miRNA signatures for various neoplasms[19], including gastrointestinal tumors such as high-risk colonic adenomas and colorectal cancers,[20] gastric cancer[21] and pancreatic cancer.[22]

We previously performed miRNA profiling in tissues from SBNET and their metastases (LN and liver), and discovered a miRNA signature able to differentiate primary tumors from normal small bowel.[23] Furthermore, we identified two miRNAs (miR-1 and miR-143-3p) significantly down-regulated in LN and liver metastases compared to primary tumors, and showed that these directly regulate *FOSB* and *NUAK2* oncogenes.[23]

Encouraged by these results, the aims of this pilot study were to evaluate the use of serum miRNAs as diagnostic biomarkers in SBNET, investigate post-operative dynamics of the dysregulated serum miRNAs, and define their usefulness in determining surgical efficacy, detecting residual disease and monitoring for disease recurrence. Importantly, circulating miRNAs distinctive of SBNET are still poorly characterized. To date, this is the first study of serum miRNA profiles in SBNET to determine biomarkers of treatment response in a longitudinal fashion. Our hypothesis was that serum miRNAs that are significantly differentially expressed in patients with SBNET may be useful for detecting residual disease (RSD) and for monitoring for recurrent disease (RCD).

METHODS

This study was approved by the Imperial College Healthcare Tissue Bank Committee and a National Research Ethics Committee (07/MRE09/54). Informed consent was obtained from all study subjects.

Study Patients

Inclusion criteria were treatment naïve SBNET patients with histologically confirmed G1 or G2 SBNET (primary tumor in the ileum); tumor stage documented according to imaging; and no severe co-morbidities or synchronous malignancy. Patient demographics and clinicopathological characteristics for each stage of the study are shown in **Table 1**. Healthy controls used were age, sex and BMI matched, and none had any history of previous malignancy. Peripheral blood samples were obtained from subjects using serum vacutainer tubes (Red Hemogard Closure 6ml tubes; BD, Franklin Lakes, NJ). Blood samples were processed within 2 hours after venesection, and spun at 2,500g for 10 minutes at room temperature. Serum was separated and stored at -80°C until required. All patients had baseline staging CT, MRI, and ^{68}Ga DOTATATE PET/CT and then follow-up imaging at 3 monthly intervals. At each clinical appointment, blood assays of CgA were performed (in-house radioimmunoassay, Neuroendocrine Tumor Supra-regional Assay Services laboratory, Imperial College Healthcare NHS Trust). Further follow-up protocols were according to European Neuroendocrine Tumor Society (ENETS) guidelines and/or clinical requirements.

Study Design

The primary end-point was identification of serum miRNAs able to detect SBNET. Whilst the secondary endpoints were to determine the effectiveness of surgical resection and post-operative dynamics at 1 day (D1), 1 week (1W), 1 month (1M) and 12 months (12M) after surgery. The study design is shown in **Fig. 1**. The study was performed in 3 stages (discovery, validation and a prospective, longitudinal assessment). All serum samples and clinicopathological data were collected prospectively.

Stage 1 – a "discovery" cohort of SBNET serum samples (n=11; locoregional disease, n=7; distant metastases, n=4; **Table 1**) vs. healthy controls (HC) (n=3) was used to identify dysregulated serum miRNAs by global miRNA profiling.

Stage 2 –a "validation" cohort of SBNET (n=33; **Table 1**) vs. HC (n=14) sera was used to confirm the findings from the Stage 1 profiling experiment by RT-qPCR using individual TaqMan Advanced miRNA assays. We also assessed the performance of each candidate serum miRNA at discriminating SBNET from HC by ROC curve analysis and AUC measurement. During this stage the newly discovered serum endogenous miRNAs or "reference genes" for normalization were also investigated.

Stage 3 – a "prospective, longitudinal assessment" of serum miRNA expression in suitable, consecutive SBNET patients undergoing surgical resection (n=12; **Table 1**) was performed with serial blood sampling: pre-operatively day 0 (D0; i.e. in the morning just prior to surgery); post-operatively D1, 1W, 1M and 12M.

Post-operatively, patients underwent standard clinical follow-up every 3 months, and disease status and response to treatment were determined by CT and/or MRI, according to the response evaluation criteria in solid tumors (RECIST 1.1), and by functional-imaging by ^{68}Ga DOTATATE PET/CT. Patients in the longitudinal cohort were therefore divided into those with no disease (ND) identifiable on imaging, and those with RSD or RCD present (i.e. regional LN involvement, liver metastases, or other distant metastases).

Statistical Analysis

The Mann–Whitney *U*-test was used to detect differences between groups. Receiver operating characteristic (ROC) curve analysis was conducted for each significantly deregulated and validated serum miRNA to understand their ability to discriminate SBNET patients from healthy controls, leading to estimates of area under the curve (AUC) with 95% confidence intervals (CI). An AUC of 1.0 represents a perfect biomarker, whereas an AUC of 0.5 indicates a result that is no better than expected by random chance (i.e. 50:50). In general, an AUC of ≥0.75 is considered a good

biomarker, whilst ≥0.9 would represent an excellent biomarker.[24] Forced-entry multivariate binary logistic regression analysis was performed, with all validated serum miRNAs entered in a single step without stepwise selection. Predicted probabilities were calculated for all analyzed samples using the logistic regression model and were used to generate the ROC curve of the combined panel and AUC. Statistical analyses were performed using SPSS software (version 20.0, IBM) or GraphPad Prism 7. P <0.05 was defined as statistical significance.

More detailed **Supplementary Methods** are available in the **Supplementary Digital Content** online.

RESULTS

Stage 1 – Discovery Cohort

Global profiling for 376 serum miRNAs revealed 4 miRNAs (miR-125b-5p; miR-362-5p; miR-425-5p; and miR-500a-5p) significantly up-regulated in SBNET vs. HC (fold change >2; $P<0.05$; **Table 2**). These serum miRNAs remained significantly differentially expressed when using the 3 or 6 miRNA GeNorm combinations for normalization, and therefore would be more robust for quantification by RT-qPCR (**Table 3**). In this cohort (n=11), CgA was elevated in 6 patients (4 patients with metastatic disease and 2 patients with locally advanced disease; median 93 pmol/L (range 19-255 pmol/L; normal level <60 pmol/L). Thus, CgA was only accurate in detecting SBNET in 55% (n=6/11; **Table 1**).

Stage 2 – Validation Cohort

Using a validation cohort, we were able to confirm miR-125b-5p, miR-362-5p, miR-425-5p and miR-500a-5p as up-regulated in SBNET vs. HC serum using the GeNorm 3-miRNA combination for normalization (**Fig. 2A-D**).

We found that all 4 miRNAs (**Fig. 3A**) had an AUC >0.75, indicating that they may be clinically useful biomarkers.[25] These included miR-125b-5p (AUC 0.796, 95% CI 0.668-0.924), miR-362-5p (AUC 0.780, 95% CI 0.634-0.925), miR-425-5p (AUC 0.759, 95% CI 0.619-0.899) and miR-500a-5p (AUC 0.812, 95% CI 0.696-0.929; **Fig. 3A**). Next, we combined the 4 serum miRNAs in order to construct a further ROC curve. This demonstrated that the serum 4-miRNA classifier had excellent accuracy for SBNET with an AUC of 0.951 (95% CI 0.895-1.00; **Fig. 3B**). Thus, the combination of miR-125b-5p, miR-362-5p, miR-425-5p, and miR-500a-5p in serum was able to accurately distinguish between SBNET and HC with the best discriminatory power (**Fig. 3B**). We found that there was no difference in serum miRNA levels between those patients with lymph-node disease only, and those with lymph-node and liver metastases (data not shown).

Stage 3 – Longitudinal Cohort

Twelve patients included in the prospective longitudinal assessment underwent surgical resection for SBNET between November 2015 and February 2017 at Imperial College Healthcare NHS Trust. All procedures were performed laparoscopically. While in 4 patients with complete resection of the loco-regional disease was achieved (R0, no distant metastases), 4 patients had primary tumor resection but remained with non-resectable level IV LN metastases and in 4 complete elimination of the locoregional disease was performed (R0) in the presence of non-resectable distant metastases. There was no morbidity or post-operative mortality. All patients were discharged home within a median of 3 days postoperatively. For the purpose of this study, patients were followed up until the end of December 2018 (i.e. >12 months). Further follow-up was according to clinical practice.

In the prospective cohort, we assessed the post-operative dynamics of the 4 serum miRNAs found to be useful for detecting SBNET disease (**Fig. 3A)**. Levels for all 4 serum miRNAs were variable at D1 and 1W post-operatively, and showed no significant difference compared to D0. Looking at follow-up at 1M and 12M, we discovered that 2 miRNAs (miR-125b-5p and -362-5p; **Fig. 4A-B**) may have potential as biomarkers for identifying residual (RSD) or recurrent disease (RCD) after surgical resection. Indeed, at 12M post-operatively, 3 patients had ND, 4 had level IV LN involvement and 4 had distant metastases (i.e. known RSD) and 1 patient developed metastatic disease in the LN (i.e. RCD).

Serum miR-125b-5p levels were significantly up-regulated in SBNET patients on D0 compared to HC (**Fig. 4A**). After surgical resection, we found that miR-125b-5p expression was variable on D1 and 1W. Interestingly, miR-125b-5p levels were significantly reduced at 1M in those with ND ($P<0.01$), whilst remaining similar to D0 levels in those with RSD (**Fig. 4A**). This was also true at 12M post-operatively (**Fig. 4A**). Interestingly, the one patient with RCD at 12M also had a

miR-125b-5p level in line with those patients with RSD and above that of HC. Importantly, at both 1M and 12M, patients with ND had miR-125b-5p levels no different to those of HC.

Serum miR-362-5p levels were significantly up-regulated in SBNET patients on D0 compared to HC (**Fig. 4B**). Again levels were variable on D1 and 1W and remained comparable to D0 levels. At 1M after resection, miR-362-5p levels were similar in those with ND and RSD. At 12M post-operatively, miR-362-5p levels were unchanged from D0 levels in those with ND. In those with RSD at 12M miR-362-5p levels were spread over a wide range (**Fig. 4B**). Interestingly, three patients with RSD at 12M had markedly high miR-362-5p levels (**Fig. 4B**), with some being much higher than those seen in the validation cohort, whilst the other three patients had levels similar to HC (**Fig. 2B**). However, looking at the patients with RSD and serum miR-362-5p levels higher than D0 in more detail did not reveal any explanation for this.

The other serum miRNAs (miR-425-5p and -500a-5p) did not change significantly neither at 1M nor 12M post-operatively in either the ND or RSD groups, despite being significantly up-regulated at D0 compared to HC (both $P<0.050$; **Fig. 4C-D**).

DISCUSSION

For the first time, we have defined the circulating miRNome of SBNET by serum miRNA profiling, and then determined serum miRNAs that may serve as biomarkers in a longitudinal fashion. We have shown that serum miRNAs have the potential to determinate completeness of surgical resection. During this study, we have developed a robust pipeline for miRNA quantification, including essential quality control steps, and therefore our findings are potentially suitable for adoption into clinical practice after further validation. We established a serum 4-miRNA combination (miR-125b-5p, miR-362-5p, miR-425-5p, and miR-500a-5p) able to differentiate SBNET from HC with an excellent AUC of 0.951. We also identified serum miR-125b-5p and -362-5p as being potentially useful for detecting RSD after surgical resection.

There have been several promising reports on circulating miRNAs as novel diagnostic and/or prognostic cancer biomarkers.[18, 26] However, studies investigating circulating miRNAs in SBNET are limited.[27-29] Li *et al.* reported the detection of miRNAs in the serum of SBNET patients, with up-regulation of miR-96, -182, -183, -196a and -200a in those treated with somatostatin analogues (SSA) compared to untreated patients or HC.[29] Furthermore, serum miR-200a was found to be up-regulated in patients with SBNET liver metastases compared to HC regardless of SSA treatment.[29] Another group found up-regulation of plasma miR-22-3p and miR-21-5p; and down-regulation of miR-150-5p were associated with metastatic SBNET, and the combination of these 3 plasma miRNAs was also highly prognostic for survival.[27] In another study, up-regulation of serum miR-7-5p in SBNET patients was confirmed, having been previously shown to be up-regulated in SBNET tissues.[30] However, no correlation between serum miR-7-5p expression and age, gender, or tumor stage was found [28].

Our study has demonstrated a serum panel of miR-125b-5p, -362-5p, -425, and -500a-5p to accurately differentiate SBNET from HC with excellent metrics (AUC of 0.951). Indeed, elevated levels of these miRNAs in patient sera may be useful for confirming the diagnosis of SBNET in cases of diagnostic dilemma, where there is clinical suspicion. In the first week post-operatively (i.e. D1 and 1W), levels for all 4 serum miRNAs were variable and did not show a reduction compared to D0.

Surgical trauma triggers an inflammatory response and this can influence circulating miRNA levels in the first post-operative week.[31] Indeed, others suggest that at least 10-14 days are required to see a reliable drop in serum miRNA expression after cancer resection compared to pre-operative levels.[32, 33]

Serum miR-125b-5p appears to correlate with disease presence after SBNET resection, and may be potentially useful for detecting of RSD or RCD. Interestingly, expression of serum miR-362-5p did not change at 1M after surgery in either the ND or RSD groups compared to D0, indicating that either it is more stable, or its production does not reflect loss of tumor burden. However, some patients with RSD at 12M were found to have extremely high levels of serum miR-362-5p, and therefore it may be a biomarker of metastatic disease or perhaps changes in the biology of the metastases, rather than primary tumor, especially since the initial discovery profiling included patients with locally advanced and metastatic disease. Thus, the monitoring of these serum miRNAs could be useful for RSD surveillance and/or RCD detection; however a much longer follow-up is needed. Furthermore, our cohort consisted of a low number of ND and only 1 patient with RCD at 12M, which did not allow a more thorough analysis of these serum miRNAs in relation to clinical phenotypes. Of note, only one study has reported prognostic utility of a miRNA panel for SBNET,[27] whilst none have assessed miRNAs suitable for monitoring SBNET patients following surgery or non-surgical treatment for confirmation of treatment response or the detection of RSD or RCD. Therefore, our study is the first to demonstrate potential utility of serum miR-125b-5p in monitoring disease status in SBNET. The use of serum miR-125b-5p as a screening tool for patients presenting with typical SBNET symptoms (e.g. abdominal pain, flushing, and diarrhoea) will require further validation.

Interestingly, the reported SBNET circulating miRNA signatures lack concordance.[27-29] Several variables, including pre-analytical, analytical, or post-analytical factors have been proposed as potentially causative for the inconsistency in miRNA studies results.[15, 34] The pre-analytical variables include the use of different biofluids (i.e. plasma [27] compared to serum [28, 29]), different methods for blood sample processing and RNA extraction, and differences in the clinicopathological characteristics of the patients. While analytical and post-analytical variables include mainly different

miRNA profiling and normalization strategies. The importance of appropriate normalization strategies is essential, considering that no consistent housekeeping miRNAs have been yet defined in biofluid-based miRNA studies. In our study, to select the most robust serum miRNA biomarkers, we analyzed the profiling results after global normalization, and normalization to 3 or 6 reference miRNA combinations (i.e. top most stable miRNAs based on the GeNorm algorithm stability score). Thus, we finally selected 4 up-regulated serum miRNAs in SBNET, which were differentially expressed irrespective of the normalization strategy, and therefore most likely to be clinically reproducible when measured by RT-qPCR. Of note, there were several down-regulated serum miRNAs (Fold Change <1; **Table 2**) in SBNET, but these did not remain significant in all 3 normalization strategies, and so were not investigated further.

We sought to validate up-regulated serum miRNAs, as the hypothesis for over-expression of miRNAs in the circulation is well-established and conveying. The tumor-related miRNAs have been proposed to be released from cancer cells into the circulation via secretion or due to cell death or necrosis. Thus, certain circulating miRNA levels would increase.[35, 36] However, since necrosis is an unlikely event in NET,[37] the alternative theory of selective miRNA secretion playing a vital role in cell-to-cell communication seems more viable.[38] However, there is a very limited overlap of dysregulated miRNAs in SBNET tissues [23] and our serum miRNA profiling. This lack of concordance of miRNA profiles has been reported previously in various cancers,[34] and other studies have noted that serum or plasma miRNA profiles may not mirror the tissue expression.[39-41] Therefore, it has been hypothesized that miRNAs dysregulated within the tumor may not be appropriate candidates for studying in biofluids.[40]

However, there has been some overlap between previous studies, as miR-7-5p has been found to be over-expressed in SBNET tissues[23, 30] and in the serum from SBNET patients.[28] Although we did not identify it in the current study. Comparing our previous tissue miRNA profiling[23] with the current serum miRNA profiling, we noticed a few miRNAs dysregulated in common (**Table 2**). However, based on our normalization strategy, these were not selected for further validation.

Importantly, the 4 serum miRNAs (miR-125b-5p; -362-5p; -425-5p; and -500a-5p) identified in our study have been previously shown to have roles in various other cancers. MiR-125b has gained a special interest in cancer research, and has been shown to be play opposing roles in various tumors.[42] MiR-125b may function either as an oncogene (oncomiR), and is seen to be up-regulated in various cancers such as colorectal, gastric, leukaemia, pancreatic and lung, or as a tumor suppressor, with down-regulation in ovarian, hepatocellular carcinoma (HCC), and breast cancer.[43-46] In addition, miR-125b-5p has been found over-expressed in serum of lung cancer patients,[40] and in the serum of breast cancer patients with tumors resistant to chemotherapy.[47] In locally advanced rectal cancer, up-regulation of miR-125b in both tissue and serum was associated with a poor therapeutic response.[42] Furthermore, serum miR-125b has been shown to distinguish non-small-cell lung cancer patients from HC, and high expression was an independent prognostic factor for survival.[48] Interestingly, Panarelli *et al.* found tissue miR-125b expression to be significantly lower in SBNET compared to appendiceal NETs.[49] Unfortunately, we did not have any serum from patients with appendiceal NET to make an assessment of the circulating levels. The up-regulation of miR-362-5p has been reported in the serum samples of patients with renal cell carcinoma.[50] Whilst in gastric cancer (GC) and HCC, it has been shown that the up-regulation of miR-362 is linked to cell proliferation and resistance to apoptosis.[51] In CRC, miR-425-5p was found to be significantly up-regulated in the serum of patients and proposed as a novel diagnostic biomarker.[52] Furthermore, its over-expression has been reported in cervical cancer, both in tissue and serum, and also able to predict poor survival in these patients.[53] It has been shown that miR-500 is significantly up-regulated in 45% of human HCC tissue samples, as well as in the serum of HCC patients, returning to normal levels after surgical resection.[54]

Study Limitations

Our study was limited by sample size, which is frequently the case in rare diseases. Furthermore, at the time of last follow-up (December 2018), none of the patients in the prospective cohort had developed disease progression and only 1 developed RCD after initially being disease free after surgery, and therefore impossible to evaluate the ability of our candidate serum miRNAs to detect these changes. Thus, our results will need to be validated in further external, larger,

multicentric studies in order to analyse the diagnostic capability of the 4-miRNA panel, and also confirm serum miR-125b as a marker of RSD after treatment. Finally, our candidate serum miRNAs were only measured in patients with known SBNET and are therefore not tumor specific, since they were not assessed in patients with non-NET small bowel disease (e.g. small bowel adenocarcinoma or Crohn's disease). In our future studies, we will endeavour to use serum from such patients as additional control groups. It is important to acknowledge that our pilot study did not adhere to all the checkpoints of the STARD (Standards for Reporting of Diagnostic Accuracy Studies) statement.[55] However, it has allowed us to be able to define the intended use and clinical role of serum miRNAs in SBNET better.

CONCLUSIONS

We have discovered a serum-based 4-miRNA signature for detecting SBNET. This miRNA panel possesses excellent metrics for improving SBNET diagnosis. We also identified that serum miR-125b-5p levels appear responsive to extent of surgical resection and able to potentially detect RSD/RCD. Whilst, serum miR-362-5p appears to be more variable and elevated levels may indicate the presence or changes in metastatic disease. The use of these serum-based miRNAs holds promising potential for clinical decision making and improving SBNET patient outcomes. It may also provide additional information when combined with other assays, such as whole-blood gene expression NETest.[9-12] Prospective validation in larger, multi-centric cohorts is now required.

FIGURE LEGENDS

FIGURE 1. Study outline. See text for details (HC, healthy controls; SBNET, small-bowel neuroendocrine tumors; ND, no disease; RSD, residual disease; RCD, recurrent disease).

FIGURE 2. Serum miRNAs are able to differentiate between healthy controls (HC) and patients with small-bowel neuroendocrine tumor disease (SBNET). Displayed are the relative expression levels for **(A)** miR-125b-5p, **(B)** miR-362-5p, **(C)** miR-425-5p, and **(D)** miR-500a-5p in SBNET (n=33) and HC (n=14). Total RNA was isolated from sera and real-time reverse-transcription quantitative PCR (RT-qPCR) was used to measure miRNA expression levels. Scatterplots are shown for each miRNA and the horizontal lines represent the median expression level and interquartile range (***P<0.001; **P<0.01).

FIGURE 3. Performance of serum miRNAs for detecting small-bowel neuroendocrine tumors (SBNET). Displayed are the receiver operating characteristic (ROC) curves and Area Under the Curves (AUC) for SBNET (n=33) vs. healthy controls (n=14). **(A)** Individual ROC curves and AUC for serum miRNAs (miR-125b-5p; miR-362-5p; miR-425-5p and miR-500a-5p) and, **(B)** ROC curve for the 4 serum miRNA combination (AUC 0.951, 95% CI: 0.895-1.000).

FIGURE 4. Serum miRNA expression assessed longitudinally in patients with small-bowel neuroendocrine tumors (SBNET) undergoing surgical resection. Displayed are the expression levels for **(A)** miR-125b-5p, **(B)** miR-362-5p, **(C)** miR-425-5p, and **(D)** miR-500a-5p. Total RNA was isolated from sera and real-time reverse-transcription quantitative PCR (RT-qPCR) was used to measure miRNA expression levels. The expression for each miRNA in healthy controls (n=14) is shown for reference, and the mean expression in healthy controls depicted by the dashed horizontal line. SBNET patients (n=12) were followed longitudinally and serum samples taken for miRNA quantification pre-operatively at day 0 (D0; n=12); then post-operatively on day 1 (D1; n=11); 1 week (1W; n=12); 1 month (1M; n=12) and 12 months (12M; n=10). At follow-up patients were grouped into those with no disease (ND), residual disease (RSD) and recurrent disease (RCD). Scatterplots are shown for each miRNA and the horizontal lines represent the median expression level and interquartile range (***P<0.001; **P<0.01; *P<0.050).

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